Neurotoxicity of the Industrial Solvent

4-Methylcyclohexanemethanol:

Involvement of the GABA Receptor

Jason Gibson

Thesis Prepared for the Degree of

MASTERS OF SCIENCE

May 2015

Committee:

Dr. Guenter W. Gross (major professor) Department of Biological Sciences
Dr. Kamakshi Gopal Department of Speech and Hearing Science
Dr. Duane Huggett Department of Biological Sciences and Waterborne Inc. Virginia

A recent chemical spill of 4-Methylcyclohexanemethanol (4-MCHM) in West Virginia left 300,000 people without water. Officials claimed that this compound is not lethally toxic, but potentially harmful if swallowed or inhaled, and can cause eye and skin irritation. Sittig's Handbook of Toxic and Hazardous Chemical Carcinogens reports high exposures from skin contact or inhalation may cause damage to the heart, liver, kidneys, and lungs, and may result in death. However, no quantitative data seem to exist and no references can be found on neurotoxicity. We have investigated the neurotoxicity of 4-MCHM using mammalian nerve cell networks grown on microelectrode arrays. Network spontaneous activity from multiple units (range 48 – 120 per network) were used as the primary readout. Individual units were followed based on spike waveforms digitized at 40 kHz (Plexon MNAP system). Dose response curves show the effective inhibitory concentration at 50 percent decrease (EC\textsubscript{50}) to average 27.4 microM SD±6.17. However, in the presence of 40 microM bicuculline, a competitive GABA\textsubscript{A} antagonist, the EC\textsubscript{50} shifts to 70.63uM SD ±4.3; implying that early, low concentration exposures to 4-MCHM involve GABA activation. Initial activity loss occurs without active unit loss (defined as 10 or more template threshold crossing per min), indicating functional interference with spike production. Full recovery has not been seen at concentrations above 130 microM, unless the culture was given bicuculline. Direct exposure to 400uM results in immediate, irreversible loss of spike production, followed by necrosis of glia and neurons.
Table of Contents

Chapter 1: Introduction ................................................................................................... 5

Chapter 2: Objective ......................................................................................................... 9
  Specific Aims ................................................................................................................... 9
  Significance of Research ............................................................................................... 10

Chapter 3: Methods .......................................................................................................... 11
  Cell Culture ................................................................................................................... 11
  Test Solutions and Applications ................................................................................... 13
  Experimental Media ...................................................................................................... 13
  Recording Stations ........................................................................................................ 14
  Data Display ................................................................................................................. 14
  Plexon ........................................................................................................................... 14
  Vernac ........................................................................................................................... 15
  Orgin ............................................................................................................................. 16
  Statistics ........................................................................................................................ 16

Chapter 4: Results ............................................................................................................. 18
  Concentration (Dose) Responses in Serum-Free Medium (DMEM) ......................... 19
  4-MCHM Dose Response in DMEM 10 (10% horse serum) ............................................. 21
  4-MCHM CRC in the Presence of Bicuculline: Involvement of GABA Receptors ......... 24
    Dose Response in DMEM with 20uM Bicuculline ..................................................... 24
    Dose Response in DMEM with 40uM Bicuculline ..................................................... 26
    CRC of 20uM Bicuculline in DMEM 4-10 ................................................................. 29
  Network Desensitization from Sequential 4-MCHM Applications ............................... 30
Further Evaluation of Repetitive Titration Effects ......................................................34
Spontaneous Recovery of Single Applications..............................................................37
Recovery from Sequential Applications .......................................................................38
Cytotoxicity ..................................................................................................................39

Chapter 5: Discussion ..................................................................................................44

References ...................................................................................................................48

Table of Figures

Figure 1.  CDC Emergency Care..................................................................................7
Figure 2.  Cell Culture ...............................................................................................11
Figure 3.  Network Grown on an MEA .................................................................12
Figure 4.  Recording Station Setup ..........................................................................14
Figure 5.  The Plexon MNAP System .......................................................................15
Figure 6.  Vernac Display .........................................................................................16
Figure 7.  Vernac Dose Response Display ................................................................18
Figure 8.  Individual and Pooled DMEM CRC .......................................................20
Figure 9.  DMEM 10 CRC ........................................................................................22
Figure 10.  Comparison of Pooled DMEM and DMEM 10 CRC ..............................23
Figure 11.  CRC Data with 20μM Bicuculline ..........................................................25
Figure 12.  CRC Data with 40μM Bicuculline ..........................................................27
Figure 13.  Comparison of CRC EC_{50} Shift ...........................................................28
Figure 14.  CRC for 20μM Bicuculline with DMEM 4-10 .........................................30
Figure 15.  Single Point Applications .....................................................................31
Figure 16.  Single Experiment of Three Dose Responses .........................................33
Figure 17.  Single Applications after a Dose Response ..............................................36
Table of Tables

Table 1. CDC Epidemiology Report ................................................................. 6
Table 2. Percent decreases of DMEM ............................................................ 19
Table 3. DMEM Percent Recovery ............................................................... 21
Table 4. DMEM 10 Percent Decreases .......................................................... 22
Table 5. 20μM Bicuculline in DMEM Percent Decreases ............................... 25
Table 6. 40μM Bicuculline Percent Decreases .............................................. 26
Table 7. Demonstrating the Separate Influences from Bicuculline ............... 28
Table 8. 20μM Bicuculline in DMEM 4-10 % Decreases .............................. 29
Table 9. Single Point Applications ............................................................... 31
Table 10. JG110 EC50 Comparison to Pooled CRCs .................................... 33
Table 11. Repetitive Dose Response Experiments ....................................... 35
Table 12. Recovery of Dose Response Summary Table ............................... 39
Table 13. Summary from Dose Response Experiments .............................. 46
Chapter 1: Introduction

The Center of Disease Control (CDC) reported over 10,000 gallons of crude 4-methylcyclohexane methanol (4-MCHM) leaked into West Virginia’s Elk River on January 9, 2014. The river is the main water supply for over 300,000 residents in 8 counties. The crude 4-MCHM contained 68-89% 4-MCHM, 5.6% propylene glycol phenyl ether (PPH), and unspecified amounts of: 4-(methoxymethyl) cyclohexanemethanol, water, methyl 4-methylcyclohexanecarboxylate, dimethyl 1,4-cyclohexanedicarboxylate, methanol and 1,4-cyclohexanediethanol (Whelton et. al, 2014). Residents quickly reported a licorice smell in their tap water. Odor detection of 4-MCHM is 500 ppm (Sullivan and Krieger, 1992). Studies show microscopic liver damage in laboratory studies at 400mg/kg/day (Toxnet, 2014). Little information concerning 4-MCHM toxicity is known and a five day water ban was removed January 19th, exposing residents to levels greater than 10ppb, which were reported concentrations of 4-MCHM in residential tap water one month after the leak (Rosen et al., 2014). The CDC and WVTASP reported levels in March 2014 to be 120uM/L from a drinking water screening (Whelton et. al, 2014).

In April 2014, a patient analysis of 369 persons who sought emergency medical treatment from crude 4-MCHM exposure revealed complaints of nausea, vomiting, gastrointestinal pain, diarrhea, and dermal irritation as the most common symptoms (Health, 2014). Table 1 has the frequency of reported symptoms from the analysis. 4-MCHM has poor water solubility. Absorption from ingestion, inhalation, and dermal exposure causes irritation of the intestinal tract, lungs, dermis, and death (Pohanish, 2008). The log KOW value is 2.55 and the elimination rate is dependent on phase 2 metabolism.
Table 1. CDC Epidemiology Report. A study of 369 individuals who sought emergency medical treatment from exposure to crude 4-MCHM. Individuals reporting having multiple symptoms seen below (Health, 2014).

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Number</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nausea</td>
<td>141</td>
<td>37.9</td>
</tr>
<tr>
<td>Rash</td>
<td>105</td>
<td>28.5</td>
</tr>
<tr>
<td>Vomiting</td>
<td>104</td>
<td>28.2</td>
</tr>
<tr>
<td>Abdominal Pain</td>
<td>90</td>
<td>24.4</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>90</td>
<td>24.4</td>
</tr>
<tr>
<td>Headache</td>
<td>81</td>
<td>21.9</td>
</tr>
<tr>
<td>Itching</td>
<td>73</td>
<td>19.8</td>
</tr>
<tr>
<td>Sore Throat</td>
<td>55</td>
<td>14.9</td>
</tr>
<tr>
<td>Eye Pain</td>
<td>54</td>
<td>14.6</td>
</tr>
<tr>
<td>Cough</td>
<td>47</td>
<td>12.7</td>
</tr>
</tbody>
</table>

Symptoms from 4-MCHM exposure of 45 patients, not listed in the April report, were diagnosed as influenza, scabies, and shingles which may have been misdiagnosed or aggravated by crude 4-MCHM exposure. These individuals were treated and discharged. In all cases the most common route of exposure was bathing, ingestion, and inhalation of crude 4-MCHM from the contaminated water (Health, 2014). The second week during the water ban the reported incidence of exposure, from emergency care records, declined for individuals seeking medical care for crude 4-MCHM exposure (Figure 1). This is most likely from avoidance of the contaminated water supply.
The West Virginia water ban lasted 5 days and was removed with an uncertainty of the consequences from further exposure to crude 4-MCHM. Toxnet reports acute exposure of 4-MCHM cause’s headache, tremors, CNS depression, narcosis, irritation, dermatitis, nausea, and vomiting. Additional studies indicate exposure causes damage to the liver, kidney, spleen, and myocardial necrosis (Toxnet, 2014). The CNS depression, narcosis effect, does not have a known mode of action although many solvents are thought to activate the GABAergic pathway. The 4-MCHM LD$_{50}$ in rats to be approximately 933mg/kg. Narcosis was seen at 800mg/kg (Hosenfeld, 1990). Other studies show exposure causes liver, kidney, heart, intestinal, dermal, and respiratory irritation, and tissue death at 50ppm (Toxnet, 2014). Exposure to 4-MCHM is primarily occupational. However, increasing incidences of public exposure, from storage leaks into water supply systems, have occurred in Oklahoma, New York, and West Virginia.
Many industrial solvents have an intoxication or central nervous system depression effect. Toluene is an example of a volatile substance that is structurally the same as 4-MCHM with the exception of the methane alcohol. Toluene acts on the mesolimbic dopamine system and through hydrogenation produces methycyclohexane (Beckley and Woodward, 2013). Similar symptoms are seen from toluene and 4-MCHM exposure. Volatile solvents such as isoflurane are generally used as anesthetics with a general assumption that they work on the GABAergic system. Being that 4-MCHM is a volatile compound with a similar structure to toluene the mostly likely mode of action for the CNS depression would be activation of the GABAergic system.

This study reports functional toxicity screening of 4-MCHM using cortical tissue on microelectrodes. Cortical tissue forms spontaneously active networks after being dissociated and seeded onto microelectrodes arrays containing 32 to 64 thin film, substrate-integrated electrodes for extracellular recordings. The study indicates that microM concentrations of 4-MCHM causes an immediate inhibitory response of spontaneous activity. The inhibitory effect is long lasting, over 10 hours, and results in a decline in channel recovery with a hyper-excitation of activity with an adverse channel loss when 4-MCHM is removed from the experimental medium.
Chapter 2: Objective

Little information is known about the neurotoxicity or neuropharmacology of 4-MCHM other than that it acts as a central nervous system depressant. The objective of this research was to quantify the functional and cytotoxicity of this compound under controlled in vitro conditions, determine time lines of possible recovery, and contribute to the elucidation of mechanisms.

Specific Aims

Specific aims were to quantify toxicity profiles of cortical networks after exposure to 4-MCHM. Experiments consisted of dose responses, short-term exposure to low and high doses, and long-term exposures of low and high doses. Analysis of these experiments will provide reliable dose-response curves, measure the speed of responses, and provide information if plasma membrane receptors were involved. The following hypotheses were tested.

Hypothesis 1: Fast acting inhibitory responses of cortical networks implicate involvement of GABA_A receptors.

Hypothesis 2: Slow responses and slow recovery in the absence of cytotoxicity implicates GABA_B receptors.

Hypothesis 3: Functional and cytotoxicity are caused by different mechanisms.
Significance of Research

4-MCHM has been leaked into water supply systems three times this past century and is used daily in industry as a solvent for removing impurities from coal (froth floatation), and is a patented chemical in air fresheners. 4-MCHM causes organ failure, myocardial necrosis, narcosis, seizures, and death (Toxnet, 2014). There is no current research about the toxicity of 4-MCHM on brain tissue. Understanding the narcosis mechanisms and the thresholds of functional and cytotoxicity will fill an information gap about the exposure limits to 4-MCHM.

Seizures caused from exposure to 4-MCHM are treated with a benzodiazepine such as diazepam or propofol (a poorly understood anesthetic thought to activate GABA) (Toxnet, 2014). Over-activation of the GABAergic system can cause immediate respiratory arrest. Identifying receptor activation of 4-MCHM may provide better treatment options for individuals exposed to 4-MCHM.
Chapter 3: Methods

Cell Culture

Cell lines cannot be used, as we require mixtures of neurons and glia cells for an appropriate representation of regions of the central nervous system. Mouse species (ICR, (CD1)) embryonic frontal cortex tissue was isolated on E17, enzymatically and manually dissociated, and seeded onto microelectrode arrays at a total cell count of 65K-85K per 100ul.

Primary Cell Culture Techniques

Figure 2. Primary cell culture techniques include: (1) A pregnant mouse E17. (2) Removing the uterus after anesthesia and cervical dislocation. (3) Separating the embryos from the embryonic sack. (4) Removing the cortical tissue. (5) Mincing and enzymatically dissociating the tissue. (6) Manual trituration and cell count. (7) Seeding onto microelectrode arrays. Picture: CNNS archives
Cortical cells seeded on microelectrode arrays (MEAs) naturally form spontaneous active networks (Gross, 1994). MEAs provide optical imaging and real-time output of extracellular recordings from numerous neurons in the network using the Plexon MNAP recording system and programs.

Network Seeded on a Microelectrode Array

Figure 3. (A) Example of a spinal cord network formed after the tissue was seeding onto a 64 channel MEA (Bodian stain; 95 day in vitro). Indium tin oxide (ITO) conductors are 10 um wide in the recording matrix (Gross et al., 1985). De-insulations are accomplished with single laser shots. (B-D) Phase contrast micrographs of living cells in a low density culture. Gold plating of the electrode terminals decreases impedance to about 0.8 megohm to allow extracellular recordings. Bar: 40 micrometer. (CNNS archives).

Cells were maintained in incubation at 37° C with 10% CO₂ to maintain the medium pH at 7.4. Medium changes (usually 50%) were performed twice weekly with additions of antibiotics only when needed. Osmolarity were maintained in a range from 300 to 340 mOsmoles.
Test Solutions and Application

4-MCHM was obtained as a 99% cis/trans mixture from TCI America. The molecular weight is 128.22 Daltons. The substance was mixed with DMSO to yield a 23mM stock solution. A new solution was made prior to each experiment.

The application method is important for reproducible results. Mixing of the solutions in medium must be done correctly. The following protocol was used.

1. Remove ~75% of recording bath medium into a 3mL syringe using the Luer port of the recording chamber.
2. Pipette a selected aliquot into the tip of a vertically oriented syringe (tip up).
3. Observe the DMSO aliquot sinking towards the plunger.
4. Pull air into the syringe to move media away from the tip.
5. Vortex the syringe contents for 5 seconds.
6. Add mixed medium back into the recording bath using the Luer port.

Experimental Media

Three recording media were used for this study: (1) DMEM (no serum), (2) DMEM 10 (10% horse serum), and (3) DMEM 4-10 (4% fetal bovine serum and 10% horse serum). During incubation cells were maintained in DMEM 10. DMEM was obtained from the HyClone Corporation and purchased through Thermo Scientific. Donor horse sera were purchased from Atlanta biologicals. Fetal bovine sera were purchased from SAFC biosciences. Sera were stored at -20\(^0\) C and thawed 1 - 3 days before preparation of media. Left over sera was not refrozen.
Recording Stations

Recording stations (Figure 4) required an MEA, baseplate, chamber, chamber cap, heating unit, inverted microscope, and pre-amps for the Plexon system. Regulation of pH required 10ml/min flow of 10% CO\textsubscript{2} mixed with air, confined by a cap with a heated ITO window that prevents condensation and allows for continuous microscopic observations. The recording system (MNAP) was obtained from Plexon Inc. (Dallas). Each preamp contains 32 channel preamplifiers and a post amplification and digitizing unit. The analog to digital conversion occurred at a frequency of 40khz, providing a resolution of 25us (Gross, 2011).

![Recording setup on microscope stage for multichannel extracellular recording.](image)

Figure 4. Recording setup on microscope stage for multichannel extracellular recording.

(1) Thermocouple, (2) Gas flow line for pH, (3) Infusion H\textsubscript{2}0 line, (4) Left 32 amplifiers, (5) Right 32 amplifiers (6) Luer port for test substance application and medium changes.

Data Display

The Plexon raster plot displays spike patterns and wave shapes of selected individual units (Figure 5 and 6), which provides readout of real-time activity for analysis of spike production mean, total activity per minute, and individual responses using NEX programing.
Figure 5. The Plexon MNAP system displays one minute activity time stamp and burst patterns from 25 wave-shape discriminated units. This is a CNNS program that combines all activity within one minute into one data point as total spike production mean spike rate. This application is effective to study influences of toxicants and pharmacological substances (picture: CNNS archives).

Vernac is a CNNS program based on Lab View. It is used in parallel with the Plexon system to display in real time the minute mean and total spike production from networks on the MEAs. Figure 5 shows the conversion of a one minute spike pattern into a single point in the Vernac display. This massive simplification is very effective for monitoring the evolution of network activity during pharmacological experiments.
Figure 6. Plexon real-time activity display. Each panel shows a spike assignment window (left) a raster display (center), and the selected wave shapes (right). Under optimal signal-to-noise ratio conditions it is possible to separate four units on one input channel in real time. (A) Raster display of a culture without any bicuculline. (B) Raster display of real-time recordings from an experiment under 40uM bicuculline in the bathing medium.

**Orgin Lab Program**

Orgin version 7 (Orgin Lab Corporation) was used for graphing and data analysis using the percent decreases from varying concentrations of 4-MCHM. This program calculated sigmoidal fits, plotted concentration response curves, and calculated the EC$_{50}$ values from individual and pooled data sets.

**Statistics**

Individual concentration response curves (CRCs) were generated from the percent activity decreases generated by sequential 4-MCHM titrations. For comparison of data from different networks with usually different initial activities, it is important to normalize all responses from each network by using the reference activity of that specific network.

This study required statistical evaluation of the influences of (a) the GABA$_A$ receptor blocker bicuculline, (b) effects of serum binding, and (c) single concentration additions of the test
compound, to networks not previously exposed, to determine system sensitization or
desensitization during sequential compound additions lasting several hours. Whereas cases (a)
and (b) involve averaged CRC values with standard deviations, case (c) requires a comparison of
an established mean with a hypothetical or single measured value (GraphPad, Statistical
guide).

Two statistical measures are required for the data presented. (1) A comparison of the means
of two groups from averaged EC$_{50}$ values with respective standard deviations, and (2) a
comparison of averages with single point data. The former requires a two-tailed t-test for the
comparison of two means, the latter a one sample t-test to compare observed (i.e. calculated)
means with an expected or measured single value. Both the tests were performed with R
statistic 3.2. In both cases the distribution of the sample means was assumed to be normal, as the
sampling of the parent population (i.e. all networks) was random.
Chapter 4: Results

Cortical tissue exposure to 4-MCHM on MEAs produces concentration-dependent inhibition. All dose response experiments were conducted with sequential applications of 4-MCHM that show concentration-dependent, stepwise activity decreases (Figure 7). To allow comparisons with other networks, the activity decreases must be normalized by calculating the percent decrease from each individual network reference. The percent decreases are used to plot dose-response curves.

Figure 7. Vernac display of total spike production per minute. Sequential applications of 4-MCHM induced an inhibitory effect on spike production. Percent decreases are calculated to generate dose response curves and recovery profiles. Despite two medium changes only 36% of the original reference activity was recovered.
Concentration (Dose) Responses in Serum-Free Medium (DMEM).

All networks selected for CRC experiments required a minimum of 20 active units and a minimal spike production mean of 100 spikes/min. Varying concentrations of 4-MCHM altered spike production of the cortical networks by reducing the individual unit spike production. Individual CRC in DMEM are summarized in Table 2 below and plotted in Figure 8. In the summary table, all concentrations used are listed horizontally and can be averaged vertically. Each horizontal row represents a single experiment. With enough data points such rows can generate a CRC. However, even single applications can be included and averaged vertically. Whereas vertical averaging provides a single pooled CRC with an SD. The plotting of data in horizontal rows generates individual CRC and respective EC\textsubscript{50} values. The individual CRC are plotted in Figure 8(A).

Table 2. Percent Decreases from DMEM Experiments. Individual dose response experiments in DMEM have an average EC\textsubscript{50} value of 27.4uM SD ± 6.17 (n=6).

*Note: 18a and 18b are separate networks from a MEP 5.

<table>
<thead>
<tr>
<th>Exp.</th>
<th>12</th>
<th>23</th>
<th>35</th>
<th>46</th>
<th>58</th>
<th>69</th>
<th>81</th>
<th>92</th>
<th>104</th>
<th>115</th>
<th>127</th>
<th>138</th>
<th>150</th>
<th>EC\textsubscript{50}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jg18a</td>
<td>10</td>
<td>37</td>
<td>52</td>
<td>-</td>
<td>60</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>34.4</td>
</tr>
<tr>
<td>JG18B</td>
<td>46</td>
<td>51</td>
<td>-</td>
<td>73</td>
<td>-</td>
<td>80</td>
<td>-</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>20.1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JG94</td>
<td>-</td>
<td>36</td>
<td>58</td>
<td>-</td>
<td>74</td>
<td>-</td>
<td>94</td>
<td>-</td>
<td>-</td>
<td>98</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>34.6</td>
</tr>
<tr>
<td>JG91</td>
<td>-</td>
<td>48</td>
<td>51</td>
<td>-</td>
<td>72</td>
<td>77</td>
<td>94</td>
<td>97</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>28.6</td>
</tr>
<tr>
<td>JG20a</td>
<td>14</td>
<td>57</td>
<td>-</td>
<td>-</td>
<td>80</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>94</td>
<td>100</td>
<td>100</td>
<td>23.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AVG</td>
<td>25.3</td>
<td>45.4</td>
<td>51.3</td>
<td>63.5</td>
<td>71</td>
<td>79</td>
<td>81</td>
<td>91</td>
<td>94</td>
<td>96</td>
<td>97.7</td>
<td>99.5</td>
<td>100</td>
<td>27.4</td>
</tr>
<tr>
<td>STDEV</td>
<td>16.6</td>
<td>8.85</td>
<td>0.58</td>
<td>7.78</td>
<td>7.3</td>
<td>4.2</td>
<td>0.7</td>
<td>2.1</td>
<td>4.04</td>
<td>1</td>
<td>0</td>
<td>6.17</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The pooled EC$_{50}$ concentration response curves (CRC) from 6 dose response experiments in Table 2 average 27.4uM (SD±6.17). When the pooled percent decreases are plotted to generate a dose response curve the EC$_{50}$ is 28.9uM. Table 3 shows the average EC$_{100}$ was 142uM (SD±38.2). The pooled data EC$_{50}$ 28.9uM was tested using a one tailed t-test against the tabulated EC$_{50}$ average of 27.4uM. The resulting p value is 0.87, indicating there is no significance difference between the averaged and pooled CRC EC$_{50}$ values.

Figure 8. (A) Individual dose responses in serum-free DMEM medium. The tabled average EC$_{50}$ is 27.4uM SD±6.17. (B) CRC generated from pooled data using vertical averaging in Table 2 with a pooled EC$_{50}$ of 28.9uM (n=6). Pooled CRC data was used to represent this data. A one tailed t-test p value (0.87) shows there is no significance difference between the tabulated percent decrease and the pooled average CRC EC$_{50}$.

Recovery percentages from dose response experiments are seen in Table 3. Additions of 4-MCHM resulted in concentration-dependent inhibition of total activity per minute. Exposure to higher 4-MCHM doses resulted in lower recovery of the network activity with higher doses. The average recovery of total spike production (n=6) was 59.6 percent SD±23.2. No experiments
had a recovery over 85 percent even after three consecutive washes to remove 4-MCHM from the bathing medium.

Table 3. Recovery percentages of 4-MCHM CRC in DMEM (no serum). No experiment had a recovery over 85%. The average recovery is 59.6% SD± 23.2 after exposure to 4-MCHM.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Date</th>
<th>Reference</th>
<th>EC&lt;sub&gt;50&lt;/sub&gt; (uM)</th>
<th>EC&lt;sub&gt;100&lt;/sub&gt; (uM)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>JG29</td>
<td>6.5.2014</td>
<td>10800</td>
<td>23.1</td>
<td>184</td>
<td>73</td>
</tr>
<tr>
<td>Jg18</td>
<td>4.18.2014</td>
<td>8800</td>
<td>34.4</td>
<td>127</td>
<td>69</td>
</tr>
<tr>
<td>JG20a</td>
<td>4.28.2014</td>
<td>1500</td>
<td>23.4</td>
<td>80.5</td>
<td>29</td>
</tr>
<tr>
<td>JG91</td>
<td>10.12.14</td>
<td>3150</td>
<td>28.6</td>
<td>173</td>
<td>71</td>
</tr>
<tr>
<td>JG94</td>
<td>10.14</td>
<td>2500</td>
<td>34.6</td>
<td>161</td>
<td>32</td>
</tr>
<tr>
<td>JG18B</td>
<td>4.18.2014</td>
<td>7300</td>
<td>20.16</td>
<td>127</td>
<td>84</td>
</tr>
<tr>
<td>AVER</td>
<td></td>
<td></td>
<td>27.3</td>
<td>142.0</td>
<td>59.6</td>
</tr>
<tr>
<td>STDEV</td>
<td></td>
<td></td>
<td>6.1</td>
<td>38.2</td>
<td>23.2</td>
</tr>
</tbody>
</table>

**4-MCHM Dose Response in DMEM 10 (10% horse serum)**

Cultures were maintained in DMEM 10 medium (10 % horse serum) during incubation. Two experimental dose responses were performed with DMEM 10 as the recording bathing medium. Evaluation of the percent decreases from the concentration exposure to 4-MCHM revealed the EC<sub>50</sub> was shifted to the right by 30uM.
Table 4 list the percent decrease from sequential applications of 4-MCHM in DMEM 10. The CRC data was plotted in Figure 9. Figure 10 shows a comparison between responses in DMEM and DMEM10 with a clear shift of the DMEM10 CRC to higher concentrations (30uM) of 4-MCHM.

Table 4. Percent decreases from dose response experiments in DMEM 10. Dose response experiments with DMEM 10 have an average EC$_{50}$ of 56.7uM SD±5.02.

<table>
<thead>
<tr>
<th>DMEM 10 Experimental % decreases</th>
<th>uM</th>
<th>12</th>
<th>23</th>
<th>35</th>
<th>46</th>
<th>58</th>
<th>81</th>
<th>92</th>
<th>127</th>
<th>161</th>
<th>207</th>
<th>219</th>
<th>241</th>
<th>299</th>
<th>345</th>
<th>EC$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>JG 102</td>
<td></td>
<td>15</td>
<td>22</td>
<td>-</td>
<td>41</td>
<td>-</td>
<td>-</td>
<td>64</td>
<td>-</td>
<td>90</td>
<td>96</td>
<td>-</td>
<td>-</td>
<td>100</td>
<td>100</td>
<td>53.2</td>
</tr>
<tr>
<td>JG 104</td>
<td></td>
<td>6</td>
<td>-</td>
<td>29</td>
<td>-</td>
<td>44</td>
<td>59</td>
<td>-</td>
<td>92</td>
<td>-</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>60.3</td>
<td></td>
</tr>
<tr>
<td>AVG</td>
<td></td>
<td>10.5</td>
<td>22</td>
<td>29</td>
<td>41</td>
<td>44</td>
<td>59</td>
<td>64</td>
<td>92</td>
<td>90</td>
<td>96</td>
<td>100</td>
<td>-</td>
<td>100</td>
<td>100</td>
<td>56.7</td>
</tr>
<tr>
<td>STDEV</td>
<td></td>
<td>6.3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>24</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3.5</td>
<td>-</td>
<td>5.02</td>
</tr>
</tbody>
</table>

Figure 9. Individual dose response curves in DMEM 10 (10% horse serum). EC$_{50}$ values for JG102 is 53.2uM and JG104 is 60.3uM. A combined average for CRC in DMEM 10 is 56.75uM SD±5.0 (Table 4).
4-MCHM dose responses conducted in DMEM 10 (10% horse serum) revealed a shift of the EC₅₀ concentrations to the right (to higher concentrations). Experimental average from DMEM 10 experiments had an EC₅₀ of 56.7uM SD±5.0, a 30uM shift to the right from DMEM medium pooled EC₅₀ of 28.9uM SD± 6.1 (Table 2), indicating serum binding. A one tailed t-test comparison of the pooled EC₅₀ value against the DMEM EC₅₀ P-value is 0.000067. The low P value rejects the null hypothesis that the two means are equal with 95% confidence interval. This indicates that serum alters the effectiveness from 4-MCHM exposure, presumably by binding this compound and lowering the free availability in the bathing medium.

A: Pooled Data CRC for DMEM 10  
B: CRC Comparison of DMEM and DMEM 10

Figure 10. Serum effects on network responses.  (A) Titration of 4-MCHM in the presence of 10% horse serum. The pooled data dose response yields an EC₅₀ concentration of 58.7uM SD± 5.02 (B) 4-MCHM dose responses comparison showing a shift to higher concentrations in the presence of serum. DMEM 10 shifts the dose response to the right by 30uM, indicating serum binding.
4-MCHM CRC in the Presence of Bicuculline: Involvement of the GABA Receptors

Bicuculline is an antagonist to the barbiturate site of GABA\textsubscript{A} and is a known epileptic drug. It has been used routinely in this laboratory to stabilize activity and to increase spike production in cortical networks \textit{in vitro}. However, bicuculline also shifts the 4-MCHM pooled dose response to substantially higher concentrations. This surprising result suggests competitive binding at the GABA\textsubscript{A} receptor. Experiments were conducted under 20\textmu M and 40\textmu M bicuculline with DMEM. Analysis of the experiments with bicuculline show that dose response shifts to the right is dependent on the concentration of bicuculline (Figure 13).

**Dose Response Experiments in DMEM with 20\textmu M Bicuculline**

Table 5 summarizes data from three 4-MCHM CRC titrations in the presence of 20\textmu M bicuculline. Networks were pre-exposed to bicuculline for a minimum of 30 minutes to establish a stable bicuculline reference. The observed changes in the inhibitory effect of 4-MCHM under bicuculline may provide a link to the mechanisms involved in its narcosis effects. Individual and pooled CRC are shown in Figure 11. Comparison between Figure 8 and 11 reveal a major shift to higher 4-MCHM concentrations from 28.1 SD ± 6.17\textmu M to 51.8\textmu M SD±5.5\textmu M. An EC\textsubscript{50} shift of 84\% reflects a major influence and strongly suggests competitive binding at the GABA\textsubscript{A} receptor. The difference is considered extremely statistically significant. An unpaired t-test of the two average EC\textsubscript{50} values yields a two-tailed P value of 0.0000192. The low P value rejects the null hypothesis that the two means are equal with a 95\% confidence interval.
Table 5. Summary of 20uM Bicuculline Does Response Experiments. Bicuculline shifts the dose response to the right indicating competitive binding at the GABA receptor. Dose response experiments with DMEM and 20uM bicuculline have an average EC$_{50}$ of 51.8uM SD±5.5.

<table>
<thead>
<tr>
<th>20uM Bicuculline in DMEM</th>
<th>% Decreases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exp.</td>
<td>12</td>
</tr>
<tr>
<td>Jg75</td>
<td>-</td>
</tr>
<tr>
<td>Jg26</td>
<td>16</td>
</tr>
<tr>
<td>JG101</td>
<td>-</td>
</tr>
<tr>
<td>AVG</td>
<td>16</td>
</tr>
<tr>
<td>STDEV</td>
<td>-</td>
</tr>
</tbody>
</table>

A: Individual 20uM Bic. In DMEM

B: Pooled Data 20uM Bic. In DMEM

Figure 11. Comparison of individual and pooled CRC data. (A) Individual CRC’s under 20uM bicuculline without serum average EC$_{50}$ is 51.8uM SD±5.5. (B) Pooled data with 20uM bicuculline in DMEM have an EC$_{50}$ of 54.02uM (n=3).
40uM Bicuculline in DMEM

To demonstrate that the bicuculline shift was concentration dependent, three experiments were conducted using 40uM bicuculline to show the agonistic effects of 4-MCHM at the GABA$_A$ receptor. Table 6 has the percent decreases of each experiment with an average EC$_{50}$ of 70.6uM SD±4.33.

Table 6. 40uM bicuculline concentration dependent percent decreases from 4-MCHM exposure. Dose response experiments with DMEM and 40uM bicuculline have an average EC$_{50}$ of 70.6uM SD±4.3.

<table>
<thead>
<tr>
<th>40uM Bicuculline % Decreases/ Concentration</th>
<th>% Decreases</th>
</tr>
</thead>
<tbody>
<tr>
<td>(uM)</td>
<td>12</td>
</tr>
<tr>
<td>JG27</td>
<td>-</td>
</tr>
<tr>
<td>JG31</td>
<td>9</td>
</tr>
<tr>
<td>JG33</td>
<td>-</td>
</tr>
<tr>
<td>AVG</td>
<td>9</td>
</tr>
<tr>
<td>STDEV</td>
<td>17</td>
</tr>
</tbody>
</table>

Individual CRC’s (Figure 12) shows an EC$_{50}$ shift to the right with an average of 70.6uM (Table 6). The large shift suggests binding to the GABA$_A$ receptor, which supports the Toxnet data that 4-MCHM is a central nervous system depressant and the data in this section suggest that the GABAergic system is involved.
Figure 12. (A) Individual 40uM bicuculline dose response experiments in DMEM (n=3) have an average EC$_{50}$ of 70.6uM SD±4.3. (B) Pooled data from the individual dose responses, yielding a single EC$_{50}$ of 72.1uM.

Figure 13 shows a summary of three pooled data CRCs with different concentrations of bicuculline (0, 20 and 40uM). The EC$_{50}$ shift is clearly evident and ranges from 28.9uM, to 54.02uM, and 72.1uM. The slopes of the pooled data CRCs are very similar. To show that the EC$_{50}$ means at 20 and 40 um bicuculline are each different from the no bicuculline condition and are also different from each other it is best to use the means and SD values derived for each condition and perform unpaired two-tailed t-test. These calculations are summarized in Table 7. P value comparison from a one sample t-test using the DMEM with 20uM bicuculline pooled EC$_{50}$ value 56.9uM against the DMEM EC$_{50}$ values is 0.00016. DMEM with 40uM bicuculline P value is 8.79x10$^{-6}$. The low P values reject the null hypothesis that the means are equal with a 95% confidence interval.
Figure 13. Bicuculline shift in EC\textsubscript{50} concentrations suggesting GABA interference. Dose responses performed in DMEM have an average EC\textsubscript{50} of 28.9uM. In the presence of 20uM bicuculline the shift is to 54.02uM. When the bathing medium contains 40uM bicuculline the EC\textsubscript{50} is shifted to 72.1uM. Data points are from pooled CRC from the corresponding Table.

Table 7. Demonstrating the separate influences from bicuculline.

<table>
<thead>
<tr>
<th>Two-tailed T test</th>
<th>P value</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native (no bicuculline) against the following</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20uM bicuculline</td>
<td>0.0025</td>
<td>Alternative hypothesis: there is significance</td>
</tr>
<tr>
<td>40um bicuculline</td>
<td>0.000019</td>
<td>Alternative hypothesis: there is significance</td>
</tr>
<tr>
<td>20um against 40um bicuculline</td>
<td>0.011</td>
<td>Alternative hypothesis: there is significance</td>
</tr>
</tbody>
</table>
CRC in 20μM Bicuculline and DMEM 4-10

DMEM 4-10 contains 4% fetal bovine and 10% horse serum. The addition of serum shifts the dose response further to the right depending on the serum concentration. The additional 4% fetal bovine serum increases the EC$_{50}$ to 137μM SD± 22.6.

Table 8. 20μM bicuculline Experiments in DMEM 4-10 Medium. Dose response experiments with DMEM 4-10 and 20μM bicuculline have an average EC$_{50}$ of 137.8μM SD±22.6.

<table>
<thead>
<tr>
<th>Exp.</th>
<th>23</th>
<th>46</th>
<th>58</th>
<th>69</th>
<th>92</th>
<th>138</th>
<th>161</th>
<th>230</th>
<th>276</th>
<th>299</th>
<th>368</th>
<th>381</th>
<th>EC$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>JG78</td>
<td>15</td>
<td>17</td>
<td>60</td>
<td>72</td>
<td>100</td>
<td>153.9</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>JG91b</td>
<td>12</td>
<td>30</td>
<td>38</td>
<td>61</td>
<td>78</td>
<td>94</td>
<td>100</td>
<td>100</td>
<td>121.8</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AVER</td>
<td>13.5</td>
<td>17</td>
<td>30</td>
<td>38</td>
<td>61</td>
<td>78</td>
<td>72</td>
<td>94</td>
<td>100</td>
<td>100</td>
<td>137.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>STDEV</td>
<td>2.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>22.6</td>
<td></td>
</tr>
</tbody>
</table>

Figure 14 CRCs represents 20μM bicuculline with DMEM 4-10. The increasing amounts of bicuculline and serum concentrations lower the inhibitory effect from 4-MCHM exposure on cortical networks. P value comparison from a one sample t-test using the average EC$_{50}$ value 137.8μM against the DMEM EC$_{50}$ values is 0.000000099. The low P value rejects the null hypothesis that the two means are equal with a 95% confidence interval.
Network Desensitization from Sequential 4-MCHM Applications

Testing for network sensitization or desensitization was done by using single point applications to native networks and plotting their values with the pooled CRC. Single applications do not fall on the pooled data CRC, but are located to the left of the the CRC (Figure 15). For example a 12uM single application (Figure 15B) aligns with the first application of sequential applications. However, a single 40uM application results in a 75% activity decrease, whereas the same concentrations after 3 sequential steps with an average of 60 minute exposure causes a 35% activity decrease. Such observation suggest a gradual desensitization occurs with exposure time to 4-MCHM. The same desensitization appears to occur with and without bicuculline (see circled data in Figure 15 A and B). This may indicate receptor down regulation after long-term (3 hours) exposure to 4-MCHM.
Table 9. Summary of single point applications.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Reference activity</th>
<th>Addition (uM)</th>
<th>% Decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>JG403</td>
<td>1000</td>
<td>230</td>
<td>100</td>
</tr>
<tr>
<td>JG53</td>
<td>6300</td>
<td>35</td>
<td>61</td>
</tr>
<tr>
<td>JG92</td>
<td>2850</td>
<td>24</td>
<td>57</td>
</tr>
</tbody>
</table>

DMEM 40uM Bicuculline

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Reference activity</th>
<th>Addition (uM)</th>
<th>% Decrease</th>
</tr>
</thead>
<tbody>
<tr>
<td>JG19A</td>
<td>1000</td>
<td>46</td>
<td>70</td>
</tr>
<tr>
<td>JG19B</td>
<td>3200</td>
<td>46</td>
<td>72</td>
</tr>
<tr>
<td>JG40</td>
<td>5500</td>
<td>12</td>
<td>6</td>
</tr>
</tbody>
</table>

Figure 15. Single point applications plotted with pooled CRC data to show desensitization of the networks from exposure to 4-MCHM. (A) Two single point applications in DMEM are plotted to the left of the pooled data CRC. This indicates desensitization of the network from increasing sequential applications during dose response experiments. (B) Two single point applications are plotted to the left of DMEM with 40uM bicuculline CRC pooled data. The data points to the left of the CRC pooled data curve indicate the network is desensitized from repetitive exposures to 4-MCHM.
To demonstrate the desensitization and the robustness of the cultures, a single network experiment is shown in Figure 16. This experiment had three repetitive dose responses with 40uM bicuculline in the bathing medium, to show that the effective concentration shift depends on bicuculline, serum concentrations, and desensitization. Experimental media consisted of: serum free DMEM, DMEM 10 (10 % horse serum), and DMEM 4-10 (4% fetal bovine and 10% horse serum). As summarized in Table 10, increasing exposures to 4-MCHM desensitized the responses during each dose response experiment. This experiment is important to show that cortical networks can be subjected to multiple manipulations over 16 hours, and reflects at least 3 separate mechanisms.

1. Serum binding of 4-MCHM, which reduces the effective concentration.
2. Antagonism of the GABA_A receptor from 40uM bicuculline.
3. Desensitization from sequential additions of 4-MCHM.
Single Experiment of Three Consecutive Dose Responses

Figure 16. Three repetitive dose responses in DMEM, DMEM10, and DMEM 4-10 all containing 40uM bicuculline. The increasing exposure time to 4-MCHM desensitized the network and required substantially more amounts of 4-MCHM to reach EC\textsubscript{50} values. DMEM (no serum) EC\textsubscript{50} is 28uM and plotted to show the native EC\textsubscript{50} value. The first exposure with 4-MCHM in DMEM and 40uM bicuculline reached an EC\textsubscript{50} of 76uM. The second EC\textsubscript{50} under DMEM 10 (10% horse serum) increased (desensitized to) 202uM. This is a 62% increases from pooled CRC data. The DMEM 4-10 (4% fetal bovine and 10% horse serum) EC\textsubscript{50} is 274uM. Each dose response was conducted after 2 washs and when a new reference line was established. (Exp. JG110).

Table 10. Comparison of JG110 and CRC pooled EC\textsubscript{50} values.

<table>
<thead>
<tr>
<th>40uM BIC</th>
<th>Application Number</th>
<th>Pooled Data EC\textsubscript{50}</th>
<th>Single Network EC\textsubscript{50} (JG110)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) DMEM</td>
<td>1</td>
<td>79.1</td>
<td>76</td>
</tr>
<tr>
<td>2) DMEM10</td>
<td>2</td>
<td>126.8</td>
<td>202</td>
</tr>
<tr>
<td>3) DMEM4-10</td>
<td>3</td>
<td>N/A</td>
<td>274</td>
</tr>
</tbody>
</table>
It is important to note that a single network with over 16 hours of experiments still provides well behaved CRC with nearly identical slopes. However, it is normally only possible under bicuculline, as a non-bicuculline containing medium would yield successively lower recoveries and make titrations questionable. Table 10 contains each individual dose response in its corresponding medium with 40uM bicuculline and the CRC pooled data to show the EC$_{50}$ concentrations are shifted after 4-MCHM exposure. However, the number of variables in this experiment are excessive and do not allow for a simple comparison of the data. Therefore in the next section multiple titrations under simpler conditions were performed to determine if desensitization continues with dose.

**Further Evaluation of Repetitive Titration Effects**

Cortical networks are normally very robust and withstand many variables and experimentation over many hours (Xia et al., 2003; Rijal-Oli and Gross, 2008; Gopal et al., 2011). However, repetitive exposures to 4-MCHM provide only partial activity and channel recoveries after medium changes. A second titration is therefore of questionable value if the recovered activity is below the accepted guidelines stated in the methods section (mean reference above 100 spikes/min and over 20 active units). To determine if the desensitization is enhanced by the second exposure, two dose response experiments were performed on the same networks under the same medium conditions (Table 11). The second titration with DMEM and 40uM bicuculline showed a shift in EC$_{50}$ values (P < .02).
Table 11. Repetitive dose response experiments.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>40uM Bicuculline decreases/concentration</th>
<th>% Decreases</th>
<th>One tailed t-test against the 40uM polled CRC: P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>JG27</td>
<td>23 46 58 69 92 103 115 150 195 230 276 322 334 368 EC50</td>
<td>JG27b 14 32 41 54 59 62 77 82 93 100 100 100 84.2 JG27b</td>
<td>0.16 (accept the null) 0.028 (reject the null)</td>
</tr>
</tbody>
</table>

The time the network remains desensitized is currently unknown. However, single point applications were performed after a new recovery reference line was established (Figure 17). The percent decrease was plotted with the individual CRC. Single applications had a similar concentration-dependent decrease after two washes, indicating that the network remains desensitized at least over 1.2 hours.
Figure 17. Single application after a titration, 2 washes, and establishment of new reference. A 40 uM application at 705 min (1.2 h after the wash; in A) induced a rapid 60% decrease. The percent decrease of the single application from the new reference shows a similar percent decrease as that obtained from the CRC (B). This implies that the desensitization has not changed after two medium changes and a one hour recovery period.
Spontaneous Recovery from Single Applications

Single applications of 4-MCHM were used to monitor spontaneous recovery. Full recovery took over 6 hours and only occurred when bicuculline was in the bathing medium. Without the presence of bicuculline, recovery was only seen after a wash, followed by a hyper-excitation above reference. The mechanism for this hyper-excitation has not been identified. The experiment in Figure 18 did not have bicuculline. The inhibitory effect lasted 23 hours. After a wash full channel recovery was seen with a hyper-excitation to 154%. A spontaneous recovery in the presence of bicuculline is shown in Figure 19. The exposure was for 18.7 hours in DMEM 10 with 40uM bicuculline. After the wash activity was too unstable to identify an activity reference, however there was a 100% channel recovery. 4-MCHM is a volatile compound. However, from the long lasting inhibitory effects seen in DMEM the compound most likely remains in the bathing medium. It is not until a medium change is performed that 4-MCHM inhibitory effects are subsided.

Figure 18. Long-term exposure to 230uM 4-MCHM. (A) The network showed a slow spontaneous recovery to only 30% of reference. After 23 hours, a medium change removed the 4-MCHM from the experimental medium causing hyper-activation often seen in 4-MCHM dose response experiments.
Figure 19. A 18.7 hour exposure to 4-MCHM in DMEM 10 with 40uM bicuculline. (A) Total activity recovered to 100% after ten hours of exposure. After a medium change the activity became irregular and unstable. Recovery is assumed to be from volatility of the compound. (B) Full channel recovery was seen after a wash. The mean activity/min loss was 15% after 13 hours of exposure to 4-MCHM.

**Recovery from Sequential Applications of 4-MCHM**

Each dose response titration provides recovery profiles seen from the percentage of spike production recovery after at least 2 washes (medium changes). Each wash contains new medium and sequential washing removes 4-MCHM from the recording chamber. Recording medium was stored for further review using liquid chromatography analysis to check for volatility of 4-MCHM. Table 12 summarizes all dose response experiments and shows recovery data. The average total activity recovery from all conditions was below 64% and the average unit recovery was under 82%.
Cytotoxicity

Cytotoxicity was not optically seen at exposures lower than 300uM. Exposures above 400uM and over 30 minutes had obvious cellular deterioration. Channel loss occurred immediately after a 12uM exposure to 4-MCHM indicating functional loss of neuronal activity. Some areas produced blebbing when exposed to milliM concentrations. Direct exposure to DMSO in control experiments, from a dribbling application caused blebbing (Figure 20C and D). Blebbing was not seen when the proper pipette application method was used. DMSO did not have significant
effects on activity or units if sequential additions lower than 1% were added to the recording chamber (Figure 20A). 20mM DMSO was reported to cause changes in muscle contractions (Velasco et al., 2003). If a single application of 20uL (1.0%) DMSO was added to the bathing medium an activity increase up to 20% was seen (Figure 20B). All experimental additions followed the pipette application explained in the methods section with lower than 0.5% DMSO per addition. No experiment had over a total of 40uL DMSO (2% in solution).

![Figure 20](image)

Figure 20. DMSO Controls. (A) The activity of a native network has little effects from sequential additions of DMSO (lower than 1%). (B) Additions of DMSO that were above 1% caused an activity increase up to a 20%. (C and D) A native network direct exposure to 1.5% (30ul in 2ml medium) DMSO using the dribbling application (not used for this study) creates blebbing. Proper mixing techniques prevents blebbing from occurring.
Some cultures with high density prevent the optical scanning for cytotoxicity. Exposures above 400uM cause immediate loss of network activity followed by necrosis of both glia and neurons. The figures below (Figures 24, 25, 26, 27) show the cytotoxicity ranging from 7 milliM to 200uM. All cells exposed to greater than 400uM had breakdown of cellular structures with glial swelling and cytoplasmic granulation. Further studies to determine whether cell death is caused by necrosis or apoptosis are in the planning stage but are not part of this Thesis.

Figure 21. DMEM 10: Time 10 minutes: NO DMSO. 7mM 4-MCHM causes immediate narcosis of cortical tissue after 10 minutes. (A) 3 large neurons. (B) After 10 minute exposure caused complete breakdown of cellular structure and extreme cytoplasmic debris. Frontal cortex culture (FC), 51 days old.
Figure 22. 3.5mM 4-MCHM causes destruction of neurons after 12 minutes. (A) Large neuron before 4-MCHM application. (B) After 12 minute exposure a complete breakdown of the cellular structure and extensive glial granulation are seen. Frontal cortex culture, 51 days old. Scale bar: 30 um.

Figure 23. 3.5mM 4-MCHM in DMEM-10 causes destruction of cortical tissue after 30 minutes. (A) Three large neurons identified by arrows. (B) 30 minutes of exposure caused complete breakdown of neuronal structures, glial swelling, and glial cytoplasmic granulation. Auditory cortex culture, 36 days old.
Figure 24. 410uM 4-MCHM exposure in DMEM-10: (A) A large multipolar neuron. (B) After a 1 hour exposure to 410uM a complete breakdown of the cellular structure of the neuron and the surrounding cells is seen. Extensive glial cytoplasmic granulation and vacuolization has developed. Auditory cortex culture, 47 days old.

Figure 25. A 215 uM exposure DMEM 10: 215uM: (A) Large multipolar neuron. (B) After 12 hours exposure to 215uM, increased glial vacuolization is seen. A putative astrocyte in the top left corner has changes shape and may be dying. Exposures of 200uM and below do not show rapid cellular breakdown. FC 42 Days old.
Chapter 5: Discussion

Human toxicity from exposure to 4-MCHM is not well established. The West Virginia chemical spill in January 2014, caused a 5 day water ban that officials removed with uncertainty about the further toxicity the 300 thousand residents would experience. The log KOW (lipid solubility coefficient of a compound) value of 4-MCHM is 2.55 (Toxnet, 2014) indicating that absorption and accumulation is likely. However, the elimination rate is dependent on phase two metabolism (glucuronidation).

4-MCHM is slightly soluble in water. For experimental purposes it was mixed with DMSO to yield a 23 mM stock solution that was remade before every experiment. DMSO controls did not alter activity when less than 1% was added sequentially with 4-MCHM. Blebbing occurs when DMSO was added to cultures by a dribbling application that is often used for hydrophilic compounds. This blebbing was caused by the cells being introduced to a much higher concentration of DMSO, because the molecule was not properly mixed with the bathing medium. Additions to the cortical networks were performed using the pipette method (explained in the methods section) for proper mixing of the compound with the recording medium. No blebbing occurred when DMSO was properly mixed (up to 2.5% in solution).

The specific aims and significance of this study was to provide neurotoxicity information about 4-MCHM exposure, develop reliable dose response curves, identify functional and cytotoxicity patterns, and allude to the mechanism of action for the fast acting microM inhibitory response, reported as narcosis in the literature.

Dose response experiments in DMEM were performed using sequential applications from a 23uM stock solution of 4-MCHM. A fast acting inhibitory response was seen within 2 minutes
after each addition and the degree of inhibition was concentration dependent. Six dose response experiments provided individual CRC with an average EC_{50} value of 27.4uM SD±6.17, with a pooled data CRC of 28.9uM. For a normal response (n=6) the average CRC value is used to represent the data set because it contains all percent decreases. The p-value (0.82) from a one tailed t-test does not show significance between the data sets.

Bicuculline (GABA\(_A\) antagonist) was used to increase activity and stabilize networks \textit{in vitro}. When 40uM bicuculline was in the recording medium, a drastic decrease of inhibition was seen from exposure to 4-MCHM. DMEM experiments containing 40uM bicuculline were used to show this bicuculline dependent shift. The pooled CRC for DMEM with 40uM bicuculline shifted the EC\(_{50}\) from 28.1uM to 72.1uM, a 44uM shift to higher concentrations. This shift strongly suggested competitive binding at the GABA\(_A\) receptor, which corresponds to the fast acting inhibitory response seen from GABA\(_A\) receptor activation. A one tailed t-test showed the bicuculline response is significantly different than the DMEM EC\(_{50}\) with a p value of 0.0000087.

To further study the competitive shift, experiments using 20uM bicuculline were used to show a concentration dependent EC\(_{50}\) shift in CRC pooled data. The CRC pooled data from DMEM with 20uM bicuculline (54.02uM: average 51.8uM SD± 5.5) fell in between the DMEM (no bicuculline) and DMEM with 40uM bicuculline CRC, showing there is a bicuculline concentration dependent shift to the right with sequential exposures to 4-MCHM (Figure 13). This strongly suggests that 4-MCHM binds to the GABA\(_A\) receptor and is the most likely mechanism for the reported narcosis effect and CNS depression stated in the literature. Two tailed t-test values in Table 7 show each data set is significantly different.

Cultures are usually maintained in DMEM 10 and experimentation using DMEM 10 medium showed a CRC increase to 56.7uM SD± 5.02, which was similar to the 20uM bicuculline shift.
Serum binds various compounds and when serum was in the recording medium during 4-MCHM exposures, the inhibitory effect was reduced. To show the effect was not only dependent on serum, bicuculline was added to show a further increase (shift to the right) when the bathing medium had varying concentrations of serum and bicuculline. Table 13 shows the pooled data CRC from these increasing concentrations.

Table 13. Summary of all dose response experiments.

<table>
<thead>
<tr>
<th>Bathing Medium</th>
<th>Pooled CRC</th>
<th>Averaged EC&lt;sub&gt;50&lt;/sub&gt;</th>
<th>Standard Deviation</th>
<th>EXP. Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMEM</td>
<td>28.1uM</td>
<td>27.4uM</td>
<td>6.1</td>
<td>6</td>
</tr>
<tr>
<td>DMEM + 20uM bicuculline</td>
<td>54.02uM</td>
<td>51.8uM</td>
<td>5.5</td>
<td>3</td>
</tr>
<tr>
<td>DMEM+ 40uM bicuculline</td>
<td>72.1uM</td>
<td>70.63uM</td>
<td>4.3</td>
<td>3</td>
</tr>
<tr>
<td>DMEM10</td>
<td>58.7uM</td>
<td>56.7uM</td>
<td>5.02</td>
<td>2</td>
</tr>
<tr>
<td>DMEM 4-10 +20uM bicuculline</td>
<td>106.8uM</td>
<td>137.8uM</td>
<td>22.6</td>
<td>2</td>
</tr>
</tbody>
</table>

Single point application decreases were plotted together with pooled CRC data for DMEM and DMEM with 40uM bicuculline. The points were located to the left (Figure 15). This is an indication that desensitization occurs from sequential additions (exposure) to 4-MCHM. This study only used first application experiments and data for analysis because a one tailed t-test indicated that a second application was significantly different than the mean (p value of 0.02). Experiments with repetitive dose responses indicate desensitization as the EC<sub>50</sub> value is shifted to the right, after the first dose response experiment (Figure 11). This desensitization effect needs to be studied further to identify possible mechanisms and how long this effect lasts. Hypothesis for this desensitization effect are:

1. Down regulation of GABA<sub>A</sub> receptors.
2. 4-MCHM is not removed from the medium, requiring less amounts during the second titration.

Two networks were used for a long-term toxicity study. After a medium change was performed the total recovery was often over 100%, while the channel recovery varied between 20 to 100%. This hyper-excitation was thought to be GABA deactivation, disinhibiting the network by causing loss of GABAergic input. However, after a new reference line was established after the first dose response experiment, an addition of 40uM bicuculline raised activity levels meaning GABA receptors were still active. Volatility of the compound was questioned. The inhibitory effects were long lasting and showed minimal volatility effects (Figure 21). Experiments with bicuculline had a faster recovery, possibly due to the disinhibition from bicuculline and possibly from interactions with excitatory receptors within the glutamate pathway (AMPA, Kinate, and NMDA). Maintaining a constant concentration of 4-MCHM was technically not possible due to the volatility of the compound. Experimental medium was frozen for future analysis using liquid chromatography to test if volatility of 4-MCHM is a cause of the apparent desensitization.

Cytotoxicity is seen within one hour at concentrations above 400uM. Cell death is immediate when cells are exposed to milliM concentrations of 4-MCHM. More research needs to be conducted using lactate dehydrogenase or another cytotoxicity assay. Lactate dehydrogenase is an enzyme released when cells undergo cytotoxic conditions and can help quantify the toxicity from 4-MCHM exposures. Low microM concentrations of 4-MCHM creates functional interference, while exposures over 400uM cause necrosis with complete inhibition of neuronal activity with no recovery. The 4-MCHM exposure of 10ppb in households one month after the leak was less than the concentrations of this study, which were in the ppm range. However, it
takes little activation of the GABAergic pathway to see behavioral changes (Reynolds and Berridge, 2001). Considering the widespread exposure of this compound more research needs to focus on behavioral changes individuals may have had from low dose exposures to 4-MCHM.

References


